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Recognition of Two New Species of Intestinal Spirochetes: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov.

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On the basis of DNA-DNA hybridization data, nine intestinal spirochete strains were grouped into five genospecies. Three of these genospecies were previously recognized *Serpulina* species, *Serpulina hyodysenteriae* (type strain, B78), *Serpulina innocens* (type strain, B256), and *Serpulina pilosicoli* (type strain, P43/6/78; previously "*Anguillina coli*"). The other two genospecies were found to be new *Serpulina* species, for which we propose the names *Serpulina intermedia* sp. nov. (with type strain PWS/A) and *Serpulina murdochii* sp. nov. (with type strain 56-150). *S. intermedia* and *S. murdochii* cells had a typical spirochete ultrastructure with 22 to 28 periplasmic flagella per cell. Various soluble sugars were growth substrates for *S. intermedia* and *S. murdochii*. During growth in basal heart infusion broth supplemented with fetal calf serum beneath an O_2-N_2 (1:99) atmosphere, cells of these new species consumed oxygen and glucose and produced H_2, CO_2, acetate, butyrate, and ethanol. The G+C content of the DNA of *S. murdochii* 56-150^T was 27 mol%, and the G+C content of the DNA of *S. intermedia* PWS/A^T was 25 mol%. In addition, a restriction fragment length polymorphism-PCR assay for the detection of intestinal spirochetes was developed. The assay was based on generation and restriction endonuclease analysis (with *Hin*fI, *Taq*I, *Sau*3A, and *Mbo*II) of a 558-bp amplicon of ribosomal DNA (rDNA) encoding 16S rRNA. The PCR amplification was specific for *Serpulina* species and *Brachyspira aalborgi*. Four restriction digest patterns were found for the five *Serpulina* species. *Hin*fI restriction differentiated *S. murdochii* and *S. innocens* from the other species. *Sau*3A and *Taq*I restrictions gave unique fragment patterns for *S. murdochii* and *S. pilosicoli*, respectively. *S. hyodysenteriae* and *S. intermedia* DNAs gave the same fragment pattern regardless of the enzyme tested. *B. aalborgi* was differentiated from the *Serpulina* species by *Mbo*II digestion of the 16S rDNA amplicon.

Swine dysentery (13) and porcine intestinal spirochetosis (36, 40) can be differentiated by their clinical signs. Swine dysentery is a severe, mucohemorrhagic diarrheal disease that sometimes leads to death. Porcine intestinal spirochetosis is a mucus-containing, nonbloody diarrheal disease which leads to a poor growth rate in young pigs. The etiological agents of these two diseases are separate species of intestinal spirochetes belonging to the genus *Serpulina*, the strongly hemolytic organism *Serpulina hyodysenteriae* (13) and the weakly hemolytic organism *Serpulina pilosicoli* (formerly called "*Anguillina coli*") (40), respectively. Some human intestinal spirochetes (1, 18, 29) have been identified as *S. pilosicoli* (25, 41). Other previously characterized spirochetes found in the gastrointestinal tracts and feces of animals and humans include two nonpathogenic species from swine, *Serpulina innocens* (31) and *Treponema succinifaciens* (5), and a human spirochete, *Brachyspira aalborgi*, which is believed to be a commensal (14). There undoubtedly are other intestinal spirochete species that either have not been cultivated or have not been characterized.

An analysis of a large collection of intestinal spirochete strains by multilocus enzyme electrophoresis (MEE) revealed great diversity among these spirochetes and led to the identification of five MEE groups (20), the three *Serpulina* spp. mentioned above and two other groups assigned the provisional species names "*Serpulina intermedius*" and "*Serpulina murdochii*" (19, 20). However, the new groups established by MEE analysis required further genetic and phenotypic analysis before they could be confirmed as new species.

More recently, MEE analysis established two additional spirochete groups (35), and comparisons of 16S ribosomal DNA (rDNA) sequences of intestinal spirochetes belonging to the seven MEE groups demonstrated that they are closely related to one another (26, 35). The highly conserved 16S rDNA sequences of these intestinal spirochetes led to a recommendation that alternative techniques, such as DNA-DNA relative reassociation, should be used to define *Serpulina* species (35).

The goals of the present study were to clearly define the proposed new species by using a recognized taxonomic tool, DNA-DNA reassociation (42), to provide descriptions of phenotypic characteristics of the new species, and to develop a discriminatory assay for *Serpulina* spp. by using a restriction fragment length polymorphism (RFLP)-PCR assay based on rDNA encoding the 16S rRNA. Based on our results, we propose the following two new species in the genus *Serpulina*: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov.

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TABLE 1. Strains of intestinal spirochetes used in this study

Taxon	Strain	16S rRNA sequence accession no.[a]	MEE group[b]	Origin	Pathogenesis	Reference(s)
S. hyodysenteriae	B78^T (= ATCC 27164^T)	M57743, U14930	I	Swine dysentery	Pathogenic for swine	13, 33
S. hyodysenteriae	R-1	U23035	I	Rhea colitis	Not pathogenic for swine	17, 27
S. innocens	B256^T (= ATCC 29796^T)	M57744, U14920	III	Swine	Not pathogenic for swine	33
S. pilosicoli	P43/6/78^T (= ATCC 51139^T)	U23032, U14927	VI	Swine colitis	Pathogenic for swine	36, 40
S. pilosicoli	WES-B	U23034	VI	Human diarrhea	Pathogenic for 1-day-old chickens and for swine	19, 37
S. intermedia sp. nov.	PWS/A^T (= ATCC 51140^T)	U23033	II	Swine diarrhea	Not pathogenic for swine	15, 21
S. intermedia sp. nov.	2818.5	None	II	Swine	Not tested	20
S. murdochii sp. nov.	56-150^T (= ATCC 51254^T)	None	V	Swine	Not tested	20
S. murdochii sp. nov.	155-20 (= ATCC 51284)	U22838	V	Swine	Not tested	20
B. aalborgi	513A^T (= ATCC 43994^T)	Z22781	VII	Human colitis	Not tested	14

[a] GenBank accession numbers for 16S rDNA sequences.
[b] MEE groups as designated by Stanton et al. (35).

MATERIALS AND METHODS

Organisms and growth conditions. The intestinal spirochetes were routinely cultivated in TGY anaerobic medium (Diagnostic Pasteur) or in brain heart infusion broth containing 10% calf serum (BHIS broth) (34) at 37 to 39°C. The organisms used included strains listed in Table 1, as well as spirochetes isolated from the blood of humans. Human strains PE90, 81/80, 28/94, RA87, 128/90, and 382/91 have been described and identified as *S. pilosicoli* previously (9, 39). The following three additional strains were identified as *S. pilosicoli* in this study: strain DOA95, isolated from a 43-year-old human immunodeficiency virus-positive man who is still living and previously had colonic spirochetosis; strain VIR94, originally isolated from a 65-year-old man who subsequently died from hepatoma; and strain FE96, isolated from a 69-year-old man who was hospitalized in a reanimation unit for hypothermia, coma, and shock and who died 2 weeks later. Spirochete cell numbers were determined by using a Petroff-Hausser counting chamber.

Phenotypic characteristics. The methods used to determine population doubling times, growth substrates, API-ZYM profiles, end products, hippurate-hydrolyzing abilities, and other culture characteristics were similar to those used in studies of *S. pilosicoli* (40) and *S. hyodysenteriae* (34). For growth substrate determinations, *S. intermedia* and *S. murdochii* cells were cultured beneath an N_2-O_2 (99:1) atmosphere in HS broth, which was basal heart infusion broth (34) supplemented with fetal calf serum (at final concentrations of 7 and 10% [vol/vol], respectively). The conditions used for electron microscopy of negatively stained bacterial cells have been described previously (40). Periplasmic flagellar numbers and cell dimensions were determined by using at least 20 cells.

DNA-DNA reassociation. *Serpulina* DNA was extracted and purified by a modified method of Marmur as described previously (33). DNA relatedness was determined by the S1 nuclease method (4) by using a previously described procedure (10). Due to the low G+C content of *Serpulina* DNA, a reassociation temperature of 55°C was used. The temperature at which 50% of the reassociated DNA was hydrolyzed by S1 nuclease (T_m) was determined. Differences between the T_m of the homoduplex (in the homologous reaction) and the T_m values of the heteroduplexes (in the heterologous reactions) (ΔT_m values) were determined in order to estimate DNA divergence.

DNA base composition. The guanine-plus-cytosine (G+C) contents of DNAs from *S. murdochii* 56-150^T and *S. intermedia* PWS/A^T were determined by thermal denaturation by using DNA from each spirochete at a final concentration of 50 μg/ml in 0.1 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The methods used to prepare DNA and buffers, to determine T_m values, and to calculate G+C contents have been described previously (22, 40). DNAs from *S. hyodysenteriae* B78^T (G+C content, 25 ± 1 mol%) and *Escherichia coli* XL-1 (50 ± 1 mol%) were used as controls.

Enzymatic amplification and detection of PCR products. The cultured intestinal spirochetes listed in Table 1 plus the human blood isolates mentioned above were tested by PCR. Bacterial cells (approximately 10^9) were harvested by centrifugation, washed twice with phosphate-buffered saline (28), resuspended in water, and heated twice at 100°C for 5 min, followed by cooling on ice for 5 min. The following two oligonucleotide primers were used: SER1 (5′-GGA-AAC GCC TCG GAT ACT GT-3′) and SER2 (5′-CCT TCC TCC TAC TTG AAC GTA-3′).

Oligonucleotides SER1 and SER2 correspond to nucleotides 720 to 733 and 1372 to 1352 in the primary structure of the *E. coli rrnB* operon corresponding to the *rrs* (16S rRNA) gene, respectively (3), or to nucleotides 599 to 618 and 1153 to 1133 of the *rrs* (16S rRNA) gene of *S. hyodysenteriae* (GenBank accession no. M57743), respectively.

Each DNA amplification mixture (50 μl) consisted of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.4), 1.5 mM $MgCl_2$, 1 mg of gelatin per ml, each oligonucleotide primer at a concentration of 1 μM, 200 mM dATP, 200 mM dTTP, 200 mM dCTP, and 200 mM dGTP. One unit of *Taq* DNA polymerase (Perkin-Elmer, Branchburg, N.J.) was used. A DNA sample corresponding to 5 × 10^7 cells was then added, and the reaction mixture was overlaid with 50 μl of mineral oil (Sigma). The PCR was performed with an Omnigene thermal cycler (Hybaid Ltd., Middlesex, United Kingdom). Twenty-five cycles consisting of 30 s of denaturation at 94°C, 45 s of annealing at 57°C, and 30 s of extension at 72°C were performed, and this was followed by an additional 10 min at 72°C. The amplification products (10 μl) were separated by electrophoresis in a 5% NuSieve (3:1) gel (FMC Bioproducts, Rockland, Maine) by using 1× TBE buffer (28), stained with ethidium bromide, illuminated by UV light, and analyzed. *Hin*fI, *Taq*I, *Sau*3A, and *Mbo*II were used according to the instructions of the supplier (New England Biolabs, Beverly, Mass).

To determine the specificity of the oligonucleotide primers for *Serpulina* species, DNA preparations from the following pathogenic, related, or intestinal bacteria were tested under the same PCR conditions: *Borrelia burgdorferi*, *Borrelia hermsii*, *Treponema denticola*, *Treponema pallidum*, *Leptospira interrogans*, *Spirochaeta aurantia*, *E. coli*, *Shigella flexneri*, *Salmonella enteritidis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Streptococcus* group D, *Staphylococcus aureus*, and *Mycobacterium tuberculosis.*

RESULTS

DNA relatedness and DNA analysis. The levels of DNA relatedness of nine strains of spirochetes are shown in Table 2. According to the criteria currently used to delineate genospecies (42), the differences in DNA relatedness and especially ΔT_m values are significant enough to define the following five species: *S. hyodysenteriae*, *S. innocens*, *S. pilosicoli*, *S. murdochii* sp. nov., and *S. intermedia* sp. nov. Within each of these species, the levels of DNA relatedness to the reference strains

TABLE 2. Levels of relative reassociation of *S. hyodysenteriae*, *S. innocens*, *S. pilosicoli*, *S. intermedia*, and *S. murdochii* DNAs

Source of unlabeled DNA	% Relative reassociation with labeled DNA from:				
	B78^T	B256^T	WES-B	PWS/A^T	56-150^T
S. hyodysenteriae B78^T	100		25	57 (7)[a]	27
S. hyodysenteriae R1	98 (2)	39 (14)		63 (7)	34 (>15)
S. innocens B256^T	29	100	22	45 (12)	66 (7)
S. pilosicoli P43/6/78^T	21	23	78	28	22
S. pilosicoli WES-B	28	29	100	26	28
S. intermedia PWS/A^T	68 (8)	44	30	100	24
S. intermedia 2818.5	68 (8)	48		80 (4)	40
S. murdochii 56-150^T	37	64 (5)		37 (15)	100
S. murdochii 155-20	28 (13)	64 (5)	29	45	79 (3)

[a] The values in parentheses are ΔT_m values (in degrees Celsius).

TABLE 3. Growth substrates for *S. murdochii* 56-150^T and *S. intermedia* PWS/A^T

Compound tested[a]	Growth response of[b]:	
	S. murdochii 56-150^T	*S. intermedia* PWS/A^T
None	0.6	0.6
D-Glucose	1.5	1.4
D-Fructose	1.0	1.4
Sucrose	1.1	1.5
N-Acetyl-D-glucosamine	1.4	1.8
Pyruvate	0.85	1.1
L-Fucose	0.9	1.1
D-Cellobiose	1.4	—
D-Trehalose	1.0	1.6
D-Galactose	—	1.1
Maltose	0.9	1.3
D-Mannose	0.9	—
Lactose	0.9	—

[a] Substrates that did not support detectable growth of either strain included D-fucose, D-glucosamine, D-ribose, D-raffinose, D-rhamnose, and D-xylose.

[b] —, no detectable growth above the background level in medium without added substrate. The values are the maximum culture optical densities at 620 nm obtained in HS broth containing the substrates. The serum concentrations in HS broth for optimum growth of *S. intermedia* PWS/A^T and *S. murdochii* 56-150^T were 7 and 10%, respectively.

ranged from 78 to 100%, with ΔT_m values of less than 4°C. Between species, the levels of DNA relatedness to heterologous reference strains ranged from 21 to 68%, with ΔT_m values of more than 5°C. The DNA homology results indicate that rhea spirochete strain R-1 is an *S. hyodysenteriae* strain and that human spirochete strain WES-B is a strain of *S. pilosicoli*, which is consistent with the identification of these strains based on MEE analysis (35).

As determined by the thermal denaturation method, the G+C content of the DNA of *S. intermedia* PWS/A^T was 25 mol% and the G+C content of the DNA of *S. murdochii* 56-150^T was 27 mol%. Control DNAs of *S. hyodysenteriae* B78^T and *E. coli* XL-1 had G+C contents of 24.2 and 50.8 mol%, respectively.

Cell morphology. Cells of *S. intermedia* PWS/A^T were 7.5 to 10 by 0.35 to 0.45 μm, and each cell had 24 to 28 flagella (12 to 14 flagella inserted at each end). Cells of *S. murdochii* 56-150^T were 5 to 8 by 0.35 to 0.4 μm, and each cell had 22 to 26 flagella (11 to 13 inserted at each end). *S. hyodysenteriae* B78^T cells had 24 to 28 flagella per cell (12 to 14 inserted at each end) and average dimensions of 6 to 8.5 by 0.35 to 0.4 μm. Although these flagellar numbers for *S. hyodysenteriae* cells are higher than the 16 to 24 flagella per cell reported previously for this pathogenic spirochete (30), we did not observe fewer than 22 flagella per cell for strain B78^T cells and for cells of other *S. hyodysenteriae* strains under our culture conditions. It was not possible to distinguish cells of *S. hyodysenteriae* B78^T, *S. intermedia* PWS/A^T, and *S. murdochii* 56-150^T based on cell dimensions, cell morphology, and numbers of flagella. *S. pilosicoli* cells generally are shorter (length, 5.3 to 7.3 μm) and thinner (width, 0.25 to 0.3 μm) than the cells of the other *Serpulina* species and have fewer flagella (8 to 10 flagella per cell) (40). *Brachyspira aalborgii* cells are 2 to 6 μm long and have eight flagella per cell (14).

Cultural characteristics. In BHIS broth under an N_2-O_2 (99:1) atmosphere, *S. hyodysenteriae*, *S. murdochii*, and *S. intermedia* had optimum growth temperatures (shortest population doubling times and highest final population densities) of 39 to 42°C. In BHIS broth at 39°C, the population doubling times of *S. hyodysenteriae* B78^T, *S. murdochii* 56-150^T, and *S. intermedia* PWS/A^T were 2 to 4 h, and the final population densities were 0.5×10^9 to 2.0×10^9 cells/ml. The spirochetes did not grow at 32 or 47°C. Cells of both new species used soluble sugars as carbon and energy sources during growth in HS broth (Table 3). The final population densities in HS broth were two- to fivefold lower than the final population densities in BHIS broth. During growth on glucose in HS broth beneath a culture atmosphere that initially contained 1% O_2, cells of *S. murdochii* 56-150^T and *S. intermedia* PWS/A^T consumed the O_2 and yielded H_2, CO_2, acetate, butyrate, and ethanol (Table 4). Hydrogen was produced in greater amounts than CO_2. In separate studies, the same products were detected with *S. murdochii* 155-20 and *S. intermedia* 2818.5, except that strain 2818.5 cultures did not produce detectable levels of ethanol. Additional phenotypic characteristics of *S. intermedia*, *S. murdochii*, and other *Serpulina* species are given in Table 5.

Differentiation of *Serpulina* species by RFLP-PCR analysis. Two primers were used to amplify DNAs from *Serpulina* strains. The first primer, SER1, was within a probe that hybridized to a region of the *rrs* (16S rRNA) gene and that has been used to differentiate *S. hyodysenteriae* from *S. innocens* (16). The second primer was chosen after we analyzed sequences of the rDNA encoding the 16S rRNA of strains of intestinal spirochetes in databases. The specificity and the sensitivity of the PCR assay were then determined by using these primers. As expected, a 558-bp band was detected after agarose gel electrophoresis of the PCR amplification products of all intestinal spirochete strains listed in Table 1. The lower limit of detection of intestinal spirochetes in the assay was approximately 20 bacteria (data not shown). No amplification products were detected with any non-*Serpulina* DNA except *Brachyspira aalborgi* DNA (data not shown).

After analyzing the *rrs* (16S rRNA gene) sequences in the databases, we found restriction sites for four enzymes to differentiate the *Serpulina* species. The 558-bp amplicon was further analyzed for restriction enzyme digestion products by using several strains of each species (Table 1). Four patterns were found for the five species of intestinal spirochetes; an example is shown in Fig. 1. *Hin*fI differentiated *S. innocens* and *S. murdochii* from the other three species (Fig. 1A, lanes 3 and 5). While *Sau*3A digestion gave a unique pattern for *S. murdochii* (Fig. 1B, lane 5), *Taq*I digestion yielded a distinguishing pattern for *S. pilosicoli* (type strain P43/6/78 and strains of human origin; Fig. 1C, lanes 6 to 15). *S. intermedia* could not be differentiated from *S. hyodysenteriae* by this RFLP-PCR assay, and this result could be related to the fact that the *rrs* se-

TABLE 4. Metabolic end products of growing cells of *S. murdochii* 56-150^T and *S. intermedia* PSW/A^T

Product[a]	Amt produced (μmol/ml of medium)[b]	
	S. murdochii 56-150^T	*S. intermedia* PWS/A^T
Acetate	25.4	14.0
Butyrate	5.2	10.3
Ethanol	1.0	1.4
CO_2	14.8	7.2
H_2	33.0	10.7

[a] The following compounds were assayed for but were not detected as products of either strain: formate, lactate, propionate, isobutyrate, valerate, succinate, butanol, and propanol.

[b] The culture medium used was HS broth supplemented with 0.4% glucose, and the initial culture atmosphere was 1% O_2–99% N_2. The oxygen in the culture atmospheres was completely consumed by both strains during growth, and the oxygen concentration was estimated to be 2 μmol/ml of culture broth. The gas yields were calculated as micromoles per milliliter and do not reflect the actual concentrations of gases in the medium.

TABLE 5. Phenotypic characteristics of *Serpulina* species[a]

Species	Hemolysis	Indole production	Hippurate hydrolysis	α-Galactosidase activity	α-Glucosidase activity	β-Glucosidase activity	No. of flagella per cell
S. hyodysenteriae	Strong	+	−	−	+	+	22–28
S. innocens	Weak	−	−	−	+/−	+	20–26
S. pilosicoli	Weak	−	+	+/−	+/−	−	8–12
S. intermedia sp. nov.	Weak	+	−	−	+	+	24–28
S. murdochii sp. nov.	Weak	−	−	−	−	+	22–26

[a] Phenotypic characteristics are based on results of tests performed in this study with *S. intermedia* PWS/A^T and 2818.5 and *S. murdochii* 56-150^T and 155.20 and on results of previous studies (7, 8, 11, 23). The symbol +/− indicates that some strains possess and other strains lack the particular enzymatic activity.

quences of these two species are very similar (26, 35). In addition, *Brachyspira aalborgi* could be differentiated from the *Serpulina* species since its amplicon was cleaved by *Mbo*II into two fragments that were 93 and 465 bp long (data not shown) and the amplicons from the *Serpulina* species were not cut by this enzyme.

DISCUSSION

The levels of DNA relatedness confirmed, as expected, the three previously delineated *Serpulina* species, *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli* (formerly called "*A. coli*"). Furthermore, DNA-DNA reassociation results also validated two new *Serpulina* species, consistent with conclusions based on MEE data (19, 20). For these new species we propose the names *S. intermedia* (previously called "*S. intermedius*") and *S. murdochii* (previously also called group B spirochetes) (19, 20). Indeed, DNA relatedness results indicate that the groups of intestinal spirochete strains defined previously on the basis of MEE results (20, 35) represent separate *Serpulina* species. Groups I, III, and VI correspond to *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli*, respectively, while group II corresponds to *S. intermedia* sp. nov. and group V corresponds to *S. mur-*

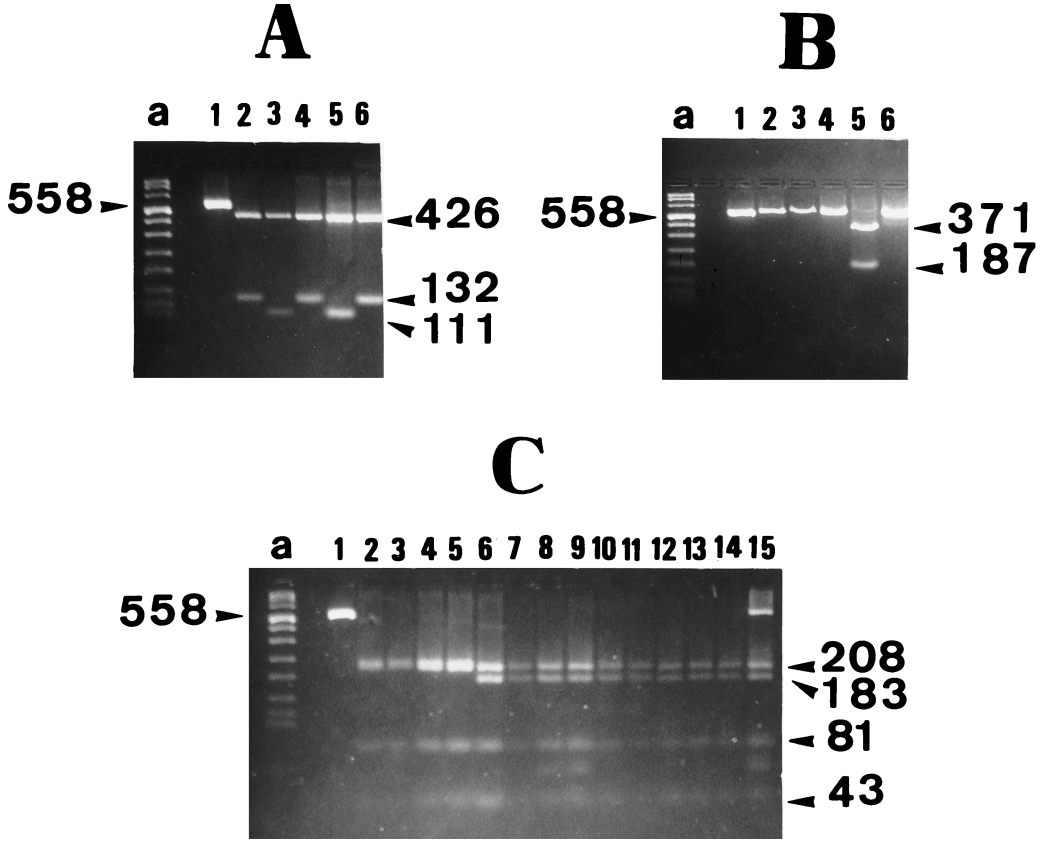

FIG. 1. RFLP-PCR analysis of *Serpulina* isolates. (A) *Hin*fI digestion of the 558-bp PCR fragment distinguishing *S. murdochii* and *S. innocens* from the other species. Lane 1, undigested fragment; lanes 2 to 6, *Hin*fI digestion patterns for *S. hyodysenteriae* B78^T (= ATCC 27164^T), *S. innocens* B256^T (= ATCC 29796^T), *S. intermedia* PWS/A^T (= ATCC 51140^T), *S. murdochii* 56/150^T (= ATCC 51254^T), and *S. pilosicoli* P43/6/78^T (= ATCC 51139^T), respectively; lane a, type VIII molecular weight markers (Boehringer, Mannheim, Germany). (B) *Sau*3A digestion of the 558-bp PCR fragment distinguishing *S. murdochii* from the other species. Lane 1, undigested fragment; lanes 2 to 6, *Sau*3A digestion patterns for *S. hyodysenteriae* B78^T (= ATCC 27164^T), *S. innocens* B256^T (= ATCC 29796^T), *S. intermedia* PWS/A^T (= ATCC 51140^T), *S. murdochii* 56/150^T (= ATCC 51254^T), and *S. pilosicoli* P43/6/78^T (= ATCC 51139^T), respectively; lane a, type VIII molecular weight markers (Boehringer). (C) *Taq*I digestion of the 558-bp PCR fragment distinguishing *S. pilosicoli* from the other species. Lane 1, undigested fragment; lanes 2 to 6, *Taq*I digestion patterns for *S. hyodysenteriae* B78^T (= ATCC 27164^T), *S. innocens* B256^T (= ATCC 29796^T), *S. intermedia* PWS/A^T (= ATCC 51140^T), *S. murdochii* 56/150^T (= ATCC 51254^T), and *S. pilosicoli* P43/6/78^T (= ATCC 51139^T), respectively; lanes 7 to 15, *Taq*I digestion patterns for *S. pilosicoli* human isolates; lane a, type VIII molecular weight markers (Boehringer).

dochii sp. nov. (Table 1). Thus, besides the strongly hemolytic organism *S. hyodysenteriae*, four weakly hemolytic species are found in the genus *Serpulina*. These species roughly correlate with the biochemical-16S rRNA groups devised by Fellström et al. (7, 8, 26), with *S. intermedia* representing their group II spirochetes, *S. innocens* representing groups IIIb and IIIc, *S. murdochii* representing group IIIa, and *S. pilosicoli* representing group IV.

S. murdochii and *S. intermedia* cells are similar in ultrastructure to cells of *S. hyodysenteriae*. These two species share a number of phenotypic properties with other *Serpulina* species. All *Serpulina* species studied use soluble sugars as growth substrates. All five *Serpulina* species consume oxygen during growth beneath an atmosphere containing 1% oxygen. A common mechanism for oxygen metabolism by *Serpulina* species is likely to be the enzyme NADH oxidase, which is present in strains of *S. hyodysenteriae*, *S. innocens*, and *S. pilosicoli* and in *S. intermedia* PWS/A^T and *S. murdochii* 56-150^T (15a, 32). Cells of the five *Serpulina* species produce more H_2 than CO_2. In *S. hyodysenteriae*, the disproportionate amount of H_2 has been attributed to an unusual mechanism for NADH-H^+ oxidation (32). It is likely that this pathway is present in other *Serpulina* species.

As additional *Serpulina* species are isolated and identified, there is a need for practical diagnostic tests to differentiate the species, especially the weakly hemolytic species, at least one of which (*S. pilosicoli*) is a demonstrated pathogen. A comparison of phenotypic properties used to identify *Serpulina* species is given in Table 5. *S. intermedia* sp. nov. and *S. hyodysenteriae* cannot be differentiated by phenotypic characteristics except for a difference in hemolysis (Table 5). *S. hyodysenteriae* and *S. intermedia* strains produce indole, while the majority of the strains of the three other species do not. Among the species which do not produce indole, hydrolysis of hippurate and lack of β-glucosidase activity allow identification of *S. pilosicoli* and the presence of α-galactosidase activity allows *S. innocens* strains to be identified (Table 5). Thus, *S. murdochii* sp. nov., which lacks α-galactosidase and α-glucosidase activities, appears to be distinct from the other species (7, 20). However, the levels of relatedness between the DNAs of the two *S. murdochii* strains and the DNA of *S. innocens* B256^T are borderline (64 to 66%) in terms of delineating species. Nevertheless, as different ways to distinguish each of these species (electrophoretic types obtained by MEE and PCR analysis followed by analysis of restriction polymorphism) are available, we propose that *S. murdochii* sp. nov. should be considered a separate species.

Several procedures have been described for identifying either *S. hyodysenteriae* (versus *S. innocens*) or *S. pilosicoli* (6, 12, 25). Our PCR assay based on the sequence of the rDNA encoding 16S rRNA and the presence of restriction sites for *Hin*fI, *Taq*I, and *Sau*3A allowed us to differentiate other *Serpulina* species, particularly *S. murdochii* (Fig. 1). *S. intermedia* could be distinguished from the other weakly hemolytic species by RFLP analysis of the 16S rDNA amplicon with all three restriction enzymes.

Description of *Serpulina intermedia* sp. nov. *Serpulina intermedia* (in.ter.me′di.a. L. fem. adj. *intermedia*, in the middle, referring to the fact that the biochemical reactivities of this organism are intermediate between those characteristically possessed by *S. hyodysenteriae* and by *S. innocens* [20]). Also called "*Serpulina intermedius*" (20). Some intestinal spirochetes referred to as "*Treponema hyodysenteriae* biotype 2" or "intermediate type" may be *S. intermedia* strains (2).

S. intermedia strains have been isolated from swine, including swine with diarrhea (7, 11). Evidence of pathogenicity after experimental infection of pigs with pure cultures has been inconclusive (15, 24). *S. intermedia* strains have been isolated from commercial poultry flocks exhibiting diarrhea and reduced production (23).

S. intermedia morphology and motility are typical of spirochetes (order *Spirochaetales*). Helical cells are 0.35 to 0.45 by 7.5 to 10 μm, generally exhibit a regular coiling pattern, and have 24 to 28 periplasmic flagella per cell (12 to 14 inserted at each cell end). Cells growing beneath an N_2-O_2 (99:1) atmosphere in stirred BHIS broth have a population doubling time of 2 to 4 h and an optimum growth temperature of 39 to 42°C and reach population densities of approximately 10^9 cells/ml. Colonies on Trypticase soy agar containing 10% defibrinated bovine blood are weakly hemolytic. The spirochete does not grow aerobically.

Substrates that support growth of *S. intermedia* PWS/A^T in HS broth containing 7% fetal calf serum include glucose, fructose, sucrose, *N*-acetylglucosamine, pyruvate, L-fucose, trehalose, galactose, and maltose. Substrates that do not support growth are D-fucose, glucosamine, ribose, raffinose, rhamnose, and xylose. In HS broth containing glucose beneath an N_2-O_2 (99:1) atmosphere, growing cells consume oxygen and yield H_2, CO_2, acetate, butyrate, and ethanol. Hydrogen is produced in greater amounts than CO_2. Strain PWS/A^T cells express NADH oxidase activity and contain the *nox* gene.

S. intermedia strains have been classified by MEE analysis (MEE group II [35]) and by biochemical tests (group II spirochetes [7, 8]). This spirochete has DNA homology levels of 57, 45, 28, and 37% with *S. hyodysenteriae* B78^T, *S. innocens* B256^T, *S. pilosicoli* P43/6/78^T, and *S. murdochii* 56-150^T, respectively. *S. intermedia* PWS/A^T can be differentiated from other weakly hemolytic intestinal spirochetes (*S. innocens*, *S. pilosicoli*, *S. murdochii* sp. nov.) by RFLP-PCR analysis of the 16S rDNA by using the enzymes *Hin*fI, *Sau*3A, and *Taq*I. The restriction patterns of the 558-bp amplicon of *rrs* contain 132- and 426-bp fragments (*Hin*fI); 43-, 81-, 208-, and 226-bp fragments (*Taq*I); and uncut 558-bp fragment (*Sau*3A).

Gram reaction negative and chemoorganotrophic. Produces indole and does not hydrolyze hippurate. Aerotolerant and anaerobic. Cells lack α-galactosidase and possess α-glucosidase and β-glucosidase activities. The G+C content of the DNA is 25 mol%. Strain PWS/A^T has all of the properties of the species and has been deposited in the American Type Culture Collection as strain ATCC 51140^T.

Description of *Serpulina murdochii* sp. nov. *Serpulina murdochii* (mur.do′chi.i. M.L. masc. gen. n. *murdochii*, of Murdoch, in recognition of work conducted at Murdoch University in Western Australia, where the type strain was identified). Previously called "group B spirochetes" or "*S. murdochii*" (19, 20). *S. murdochii* strains have been isolated from intestinal contents of healthy swine and rats (38). The spirochete is not considered a pathogen.

S. murdochii morphology and motility are typical of spirochetes (order *Spirochaetales*). Substrates that support growth of *S. murdochii* 56-150^T in HS broth containing 10% fetal calf serum include glucose, fructose, sucrose, *N*-acetylglucosamine, pyruvate, L-fucose, cellobiose, trehalose, maltose, mannose, and lactose. Substrates that do not support growth are galactose, D-fucose, glucosamine, ribose, raffinose, rhamnose, and xylose. Additional ultrastructural, motility, and growth characteristics of this spirochete are essentially the same as those reported above for *S. intermedia.*

S. murdochii strains have been classified by MEE analysis (MEE group V [35]). Type strain 56-150 has DNA homology levels of 27, 66, 22, and 24% with *S. hyodysenteriae* B78^T, *S. innocens* B256^T, *S. pilosicoli* P43/6/78^T, and *S. intermedia* PWS/A^T, respectively. *S. murdochii* 56-150^T can be differenti-

ated from other *Serpulina* species (*S. hyodysenteriae*, *S. innocens*, *S. pilosicoli*, *S. murdochii* sp. nov.) by RFLP-PCR analysis of the 16S rDNA gene by using the enzyme *Sau*3A. The *Hin*fI and *Sau*3A restriction patterns of a 558-bp amplicon of *rrs* contain three fragments (426, 111, and 21 bp) and two fragments (371 and 187 bp), respectively.

Gram reaction negative and chemoorganotrophic. Does not produce indole and does not hydrolyze hippurate. Aerotolerant and anaerobic. Cells lack α-galactosidase and α-glucosidase activities and possess β-glucosidase activity. The G+C content of the DNA is 27 mol%. Strain 56-150^T has all of the properties of the species and has been deposited in the American Type Culture Collection as strain ATCC 51254^T.

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